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HPLC Analysis of Retinoids Extracted from the Planarian, *Dugesia japonica*

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ABSTRACT—Retinoids extracted from the planarian, *Dugesia japonica* were analyzed by high-pressure liquid chromatography (HPLC). All-*trans* retinal, all-*trans* retinol, and all-*trans* retinyl ester were detected in the extracts from the head and tail pieces of the worm, while 11-*cis* retinal was detected in the extracts from the head pieces. The amounts of all-*trans* retinal, 11-*cis* retinal and all-*trans* retinol including the retinyl ester were 0.1–1.1, 0.11–0.19, and 20–50 pmol/head, respectively. The planarian contained many oil-droplets which emitted the green-yellow fluorescence probably derived from retinol and retinyl ester. These results suggest that the planarian contains all-*trans* retinol and the retinyl ester in oil-droplets and 11-*cis* retinal as the chromophore of the visual pigment in the eye.

INTRODUCTION

The planarian is one of the lowest metazoans. The eye of the planarian consists of pigmented cells and photoreceptors of a microvillar type [1]. Extracellular microelectrode recordings from the eye of the planarian *Dugesia tigrina* suggested the presence of a rhodopsin-like photopigment whose absorption maximum was at about 508 nm [2]. Spectral phototaxis experiments showed the sensitivity maximum of the planarian eye (*Planaria lugubris*) at about 475 nm [3] and 530 nm for *Dendrocoelum lacteum* [3]. The differences in those maxima may have been caused by a contribution from the dermal photoreceptors [2], an effect of screening pigments [2] or, perhaps, simply a species difference. At present, the visual pigment of the planarian has hardly been investigated by spectrophotometric methods, because the worm contains so little visual pigment. In histochemical experiments, it has been reported that the photopigment of the planaria (*Dugesia japonica*) is a chromoprotein which possesses retinaldehyde as the chromophore [4]. Recently an immunochemical study suggested the presence of rhodopsin-like protein in the head of the planarian *Dugesia japonica* by use of anti-frog-rhodopsin rabbit IgG [5].

Although 11-*cis*-retinal is the most ubiquitous as the chromophore in the vertebrate and invertebrate rhodopsin [6], a variation in the chromophore of visual pigment is found in other species: 11-*cis* 3-dehydroretinal is found in many fresh water vertebrates [7, 8] and invertebrates [9, 10]; 11-*cis* 3-hydroxyretinal is found in the insects [11]; and 11-*cis* 4-hydroxyretinal is in a bioluminescent squid [9]. In addition all-*trans* retinal and 13-*cis* retinal are seen in *Halobacterium halobium* [12]. It is unknown whether or not the chromophore of the visual pigment of the planarian is 11-*cis* retinal. The purpose of this study is to estimate the configuration of the chromophore of the planarian visual pigment by high-pressure liquid chroma-

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tography (HPLC) analysis of retinoids extracted from the worms. Our results indicate that 11-*cis* retinal is one of the most plausible candidates for the chromophore.

MATERIALS AND METHODS

Materials

The planarian worms, *Dugesia japonica* were collected from streams in the suburbs of Kyoto city (Kyoto prefecture, Japan) and Kiryu city (Gunma prefecture, Japan). Kyoto worms were maintained by feeding on fresh beef livers, and used for extractions of retinoids from their whole bodies or both the head pieces (anterior part containing the eyes) and the tail pieces (the tissues without the head). Head pieces of kiryu worms were stored as frozen materials and used for extraction of retinoids.

Extractions of retinoids

Usually, retinoids were extracted from the fresh or frozen head pieces by the oxime method which was developed to extract the retinal from biological materials as retinaloximes (*syn*- and *anti*-forms) in the original isomeric configuration without thermal isomerization [13, 14]. The planarian samples were homogenized in a solution of 100 mM NH_2OH (pH 7.2) and methanol (final concentration of methanol was 60–70%) using a homogenizer (Physcotron NS-50, Nichion Irikakikai Seisakusho Co. Ltd., Japan). The homogenate was mixed with dichloromethane and n-hexane (1:2, vol/vol), shaken vigorously and centrifuged at 2,500 r.p.m. for 15 min. The upper layer (dichloromethane/hexane layer) was collected. This extraction was repeated three times. The collected solution was stored as extracts of retinoids.

In a few cases, the planarian samples were freeze-dried for hexane extraction of retinoids. The freeze-dried samples were shaken vigorously in hexane solvent and centrifuged at 2,500 r.p.m for 15 min. The supernatant was collected and hexane extraction was repeated 3 times. The collected solution contained retinol and retinyl ester. Precipitates were gently aspirated to evaporate hexane solvent and served for the extraction of retinoids by the oxime method as described above. The obtained solution (dichloromethane/hexane extract) contained retinaloxime and retinyl ester. After evaporating solvents of the extracts mentioned above, the residues were dissolved in 50 μl of hexane/diethylether/ethanol (90/10/0.1, vol/vol) and analyzed by HPLC. All procedures were carried out under dim red light.

Detection of retinoids in the samples

Extracts of retinoids from the planarian samples were analyzed by the HPLC method as reported previously [15]. An HPLC system equipped with a 4.6×250 mm column of YMC-Pack A-003-3 SIL (Yamamura Chemical Labo. Co. Ltd., Japan) and a pump (TRI ROTER, JASCO, Japan) was used. The eluent was a mixture of n-hexane, diethylether and ethanol (90:10:0.1, vol/vol) and was used at the flow rate of 1.3 ml/min for 50 min. The absorbances of the fractions at 350 nm and at 280–500 nm were measured with a detector UVDEC-100-III (JASCO, Japan), and with a multi-wavelength detector MULTI-340 (JASCO, Japan), respectively. The measurement with MULTI-340 was carried out in order to obtain the absorption spectra of the fractions over the wide range of wavelengths, although the sensitivity of the detector was less than that of the UVIDEC-100-III detector.

Quantities of several retinoids were estimated from their abosrption coefficients and the peak areas of known amount of standard retinoids. The fractions of 2–8 min (retention time) under our HPLC conditions were used as the sample of retinylester. The solvent was evaporated from the fractions and the residue was incubated in 6% KOH-methanol solution at 25°C for 1 hr for saponification. The amount of retinyl ester was calculated from that of retinol produced by the saponification.

Observation of fluorescent images of oil-droplets in planarian tissues

The planarian worm of 5 mm in length was put on non-fluorescent slide glass, covered with a thin coverslip and spread by the squash method. The fluorescent images of the oil-droplets in the spread

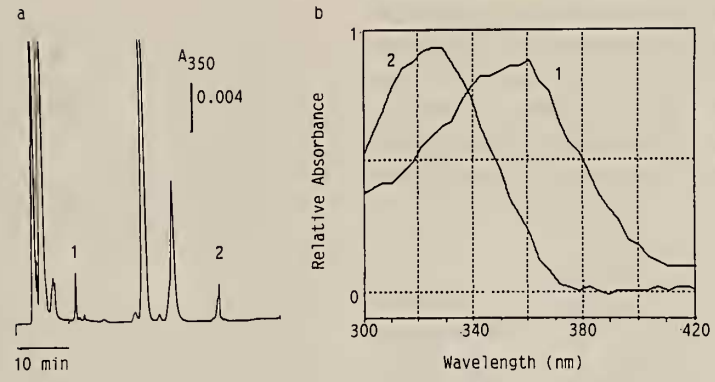

FIG. 1. (a) HPLC profile of retinoids extracted from 18 bodies of planarian worms. (b) Absorption spectra of peak fractions indicated by numbers 1 and 2. The worms were dark-adapted overnight in aged tap water at 20°C. Extractions were carried out by the oxime methods.

specimen were observed using a flouorescence microscope (Olympus inverted-microscope, IMT-2, equipped with Olympus incident-illumination type fluorescence apparatus, IMT2-RFL). The specimen was excited by light (50 W halogen lamp) passing through an excitation-filter (UG-1) and observed through a filter (L420) and a dichroic mirror (DM 400). The photograph of fluorescent images was taken using the color film (Fujichrome DX 400D).

RESULTS

Retinoids detected in the planarian

Figure 1a shows an HPLC profile of the extract of retinoids from the whole bodies of 18 planarian worms of about 10 mm in length. This figure was obtained by recording the absorbaces of the fractions at 350 nm with the UVIDEC-100-III detector. Numbers 1 and 2 indicate peaks close to the retention time of standard *syn* all-*trans* retinaloxime and all-*trans*-retinol, respectively. Two big peaks between 1 and 2 are not identified. The relative absorption spectra (300–420 nm) of the fractions corresponding to peaks 1 and 2 were obtained by the MULTI-340 detector, indicated as curves 1 and 2 in Fig. 1b, respectively. The absorption maxima of curves 1 and 2 clearly match those of the standard *syn* all-*trans* retinaloxime (λ_{max}=358 nm) and all-*trans* retinol (λ_{max}=325 nm), respectively. The fractions between 2 and 8 min, which contain retinyl esters, were collected and saponified as described in Materials and Methods. Then the material obtained after the saponification was analyzed by HPLC. From these analyses, the amounts of all-*trans* retinal, all-*trans* retinol and all-*trans* retinyl ester were calculated as 2.5, 14.7 and 99.4 pmol/body, respectively.

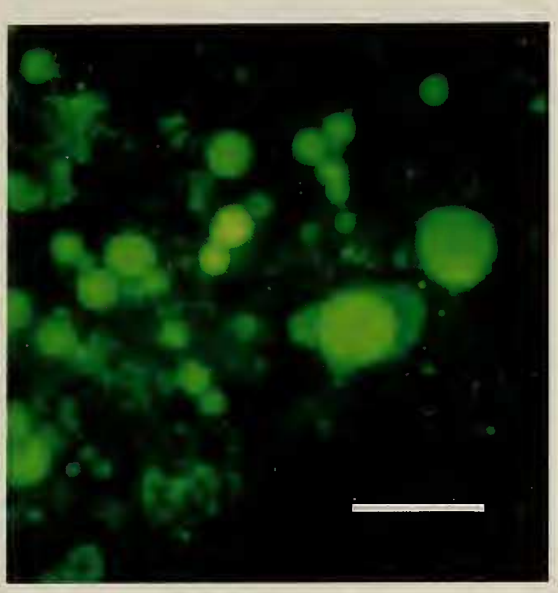

FIG. 2. Fluorescent micrograph of oil-droplets in the planarian body. The specimen was obtained by the squash methods. Bar=50 μm.

Figure 2 shows fluorescent images of several

oil-droplets in the planarian specimens obtained by the suqash method. The light color of fluoresence was green-yellow suggesting the presence of retinol and/or retinyl ester. Probably, the planarian worms store the all-*trans* retinyl ester in oil-droplets, because the worms contain large amounts of the retinyl esters, as mentioned above (more than 85 mol% of total retinoids).

Figure 3 shows HPLC profiles of extracts from the head (a) and the tail (b) pieces of 28 planarians. Peaks numbered 1 and 2 are corresponding to *syn* all-*trans* retinaloxime and all-*trans* retinol, respectively, as estimated from their retention times and abosorption spectra (data not shown). Thus all-*trans* retinal and all-*trans* retinol were found in the head and tail pieces of the planarian.

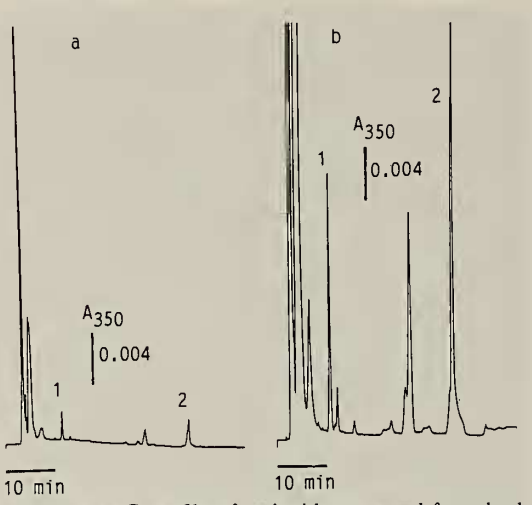

Fig. 3. HPLC profile of retinoids extracted from both the head (a) and tail (b) pieces of 28 planarian bodies. The worms were dark-adapted overnight in aged tap water at 20°C, separated into head and tail pieces under dim red light and were extracted by the oxime methods.

Retinals in the planarian head pieces

Figure 4a shows an HPLC profile of the extract of retinoids from head pieces. The head pieces were cut off from the 600 bodies of the planarian under room light, then dark-adapted overnight in aged tap water at about 20°C and stored at −20°C until use. The chromatogram was obtained by recording the absorbances of the fractions at 350 nm with the MULTI-340. Retention times of

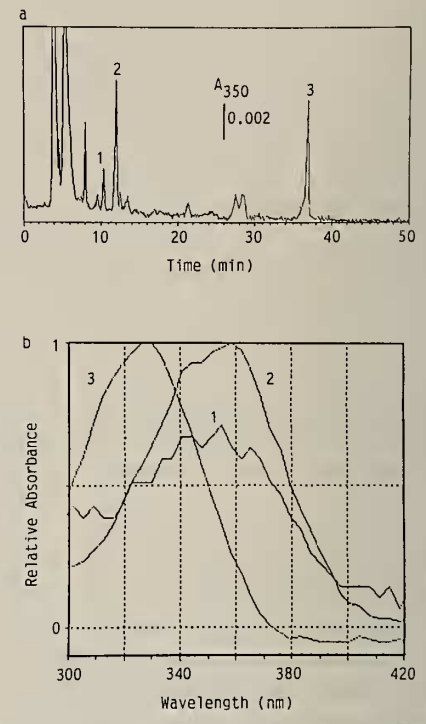

Fig. 4. (a) HPLC chromatogram of retinoids extracted from head pieces of the planarian worms. (b) Absorption spectra of peak fractions indicated by numbers 1, 2 and 3. The planarian head pieces were separated from 600 bopdies of the planarian worms under room light, then dark-adapted overnight in aged tap water at 20°C and stored at −20°C until use. Extractions were carried out by the oxime methods.

peaks 1, 2 and 3 are close to those of standard *syn* 11-*cis* retinaloxime, *syn* all-*trans* retinaloxime and all-*trans* retinol, respectively. The relative absorption spectra of fractions corresponding to peaks 1, 2 and 3 are represented as curves 1, 2 and 3 in Figure 4b respectively. Curves 2 and 3 are due to the absorption spectra of *syn* all-*trans* retinaloxime and all-*trans* retinol, respectively, as indicated in Figure 1. Curve 1 seems to be corresponding to the absorption spectrum of *syn* 11-*cis* retinaloxime, because the shape of curve 1 is different from that of surve 2 due to *syn* all-*trans* retinaloxime. The amounts of all-*trans* retinal, 11-*cis* retinal and all-*trans* retinol including the retinyl ester in different preparations were 0.1–1.1, 0.11–0.19 and 20–50 pmol/head, respectively.

In order to elucidate whether or not 11-*cis* and

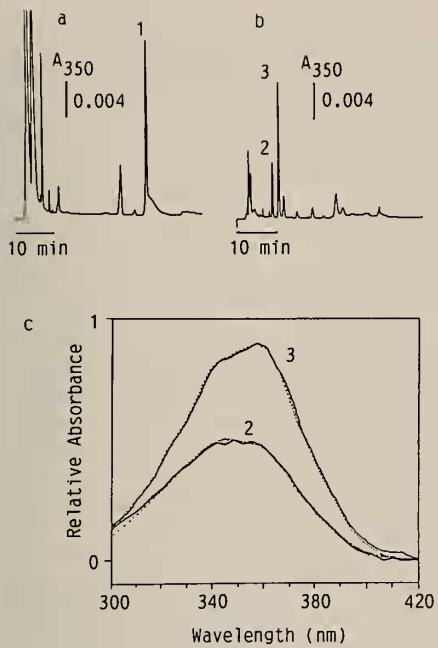

FIG. 5. (a) HPLC chromatogram of hexane extract from freeze-dried sample of the planarian heads prepared as mentioned in Fig. 4. (b) HPLC chromatogram of retinoids extracted by the oxime method from the residues after the hexane extraction. (c) Absorption spectra of peak fractions indicated by numbers 2 and 3 (solid lines) and standard syn 11-*cis* and syn all-*trans* retinaloximes (dotted lines).

all-*trans* retinals were bound to any protein in the planarian tissues, we carried out an experiment as follows. The hexane extract from the freeze-dried samples of planarian heads was analyzed by HPLC. As shown in Figure 5a, the peak of all-*trans* retinol (peak 1) was quite large, while the peaks due to 11-*cis* and all-*trans* retinals were not found. Figure 5b is an HPLC profile of retinoids extracted from the residues by the oxime methods after the hexane extraction. The figure indicates substantial peaks, numbered 2 and 3, corresponding to *syn* 11-*cis* retinaloxime and *syn* alll-*trans* retinaloxime, respectively. Figure 5c indicates the relative absorption spectra of fractions corresponding to peaks 2 and 3 as curves 2 and 3. These spectra are very similar to those of standard *syn* 11-*cis* retinaloxime and *syn* all-*trans* retinaloxime (indicated by dotted lines), respectively. The early fractions (2–8 min) in Figure 5a have peaks which are much larger than those in Figure 5b, indicating that retinyl esters were mostly extracted by the hexane extraction. Thus hexane extracted almost all of the all-*trans* retinol along with retinyl esters in the planarian tissues leaving 11-*cis* and all-*trans* retinals.

DISCUSSION

As shown in Figure 1, retinoids extracted from the homogenates of 18 bodies of the planarian were composed of all-*trans* retinal (2.1 mol%), all-*trans* retinol (12.6 mol%) and all-*trans* retinyl ester (85.2 mol%). Retinyl ester is probably a main storage form of retinoids in the planarian body and seems to exist in the oil-droplets, which emit the green-yellow fluorescence as seen in Figure 2. It has long been known that vitamin A is stored mainly as retinyl ester in the livers of a numbers of vertebrate species and that these retinyl esters are present in oil-droplets of the liver fat-storing cells [16]. The planarian has oil-droplets in the fixed parenchymal cells [17]. Probably the planarian is capable of storing retinol in ester form in the parenchymal cells.

The hexane extract from the freeze-dried planarian heads contained almost all of the all-*trans* retinol and the retinyl ester in the tissues. However, all-*trans* and 11-*cis* retinals could not be detected in the extract. Both of the retinals were extracted from the residues, after the hexane extraction, as the oximes. It is well known that retinals combined with amino group of a protein in tissues (e.g. vertebrate and invertebrate retinas) are not extracted by hexane. Therefore the retinals in the planarian may be bound to an unidentified proteins in the tissues.

All-*trans* retinal was detected in extracts of both head and tail of 28 planarians (see Fig. 3), while 11-*cis* retinal was detected in extracts derived from 600 planarian head pieces (see Fig. 4 and Fig. 5). It is reasonable to infer that the 11-*cis* retinal was derived from the chromophore of visual pigment of the planarian photoreceptor. The eye of the planarian used in this experiment is assumed to be a sphere of about 90 μm in diameter. The rhodopsin concentration of invertebrate photoreceptors is

0.3–0.4 mM [18]. We can estimate the amount of the chromophore per planarian eye, if the eye is assumed to be filled with microvilli which contain rhodopsin in the concentrations of 0.3–0.4 mM. The calculated value of the amount of chromophore was 0.11–0.15 pmol per eye, which was close to the amount of 11-*cis* retinal indicated in this experiment i.e., 0.11–0.19 pmol per head.

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